Supporting Information

The GenK-catalyzed C-6' Methylation in the Biosynthesis of Gentamicin: Isolation and Characterization of a Cobalamin-dependent Radical SAM Enzyme

Hak Joong Kim, Reid M. McCarty, Yasushi Ogasawara, Yung-nan Liu, Steven O. Mansoorabadi,

Jake LeVieux, and Hung-wen Liu*

Division of Medicinal Chemistry, College of Pharmacy, and Department of Chemistry and Biochemistry, University of Texas at Austin, Austin, Texas 78712, USA

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S1. GENERAL

Protein concentrations were determined by Bradford assay[1] using bovine serum albumin (BSA) as the standard. The relative molecular mass and purity of enzyme samples was determined using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The general methods and protocols for recombinant DNA manipulations were as described by Sambrook et al.[2] DNA sequencing was performed at the Core Facilities of the Institute of Cellular and Molecular Biology, University of Texas at Austin. The MS analyses were carried out at the Mass Spectrometry and Proteomics Facility of the Department of Chemistry and Biochemistry, University of Texas at Austin.

S2. Experimental Procedures

2.1. *Materials and Abbreviations*

Adenosine triphosphate (ATP), benzyl viologen (BV), 5′-deoxyadenosine (5′-dAdo), 1-fluoro-2,4-dinitrobenzene (DNFB), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), gentamicin X_2 ($GenX_2$), geneticin (G418), hydroxocobalamin (HOCbl), 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), isopropyl β-D-1-thiogalactopyranoside (IPTG), Luria-Bertani (LB), methyl-cobalamin (MeCbl), methyl viologen (MV), nicotinamide adenine dinucleotide phosphate hydrate (NADPH), phenylmethysulfonyl fluoride (PMSF), *S*-adenosylhomocysteine (SAH), trichloroacetic acid (TCA), tris(hydroxymethyl)aminomethane hydrochloride (Tris•HCl), and all other reagents were obtained from commercial sources. *Escherichia coli* DH5α cells were bought from Bethesda Research Laboratories (Muskegon, MI). The vector pET24b(+) and enzyme KOD DNA polymerase were purchased from Novagen (Madison, WI). DNA modifying enzymes (for restriction digestion and ligation), PCR primers, and the overexpression host *E. coli* BL21 star (DE3) were acquired from Invitrogen (Carlsbad, CA) and New England Biolabs (NEB, Beverly, MA). Luria Bertani (LB) media are products of Difco (Detroit, MI) or Fisher Scientific (Pittsburgh, PA). Pre-stained protein markers were bought from NEB. Kits for DNA gel extraction and spin miniprep were obtained from Qiagen (Valencia, CA). All reagents for SDS-PAGE and Amicon and Microcon YM-10 filtration products were purchased from Bio-Rad (Hercules, CA) and Millipore (Billerica, MA), respectively.

2.2. *Cloning and Expression of Micromonospora echinospora GenK*

The gentamicin producer *M. echinospora* (NRRL 2953) was obtained from the Agricultural Research Service of the US Department of Agriculture as a lyophilized sample. It was inoculated into 10 mL of tryptic soy broth (TSB) starter culture,[3] which was incubated overnight at 30 °C with shaking at 250 rpm. The following day, 0.1 mL of starter culture was inoculated into 10 mL of fresh TSB, and the resulting culture was incubated at 30 ^{o}C for 72 hr. Chromosomal DNA was extracted from 1.5 mL of this culture using a Qiagen DNeasy tissue kit. The *genK* gene was PCR amplified from the isolated *M. echinospora* DNA with the following primers: 5′-CAACATATGAACGCGCTGGTGGCAGC (forward) and 5′-TAACGAATTCAGTGGGAAACCGCCTCGG (reverse), and cloned into a pCR:blunt vector (Invitrogen) prior to excision with *NdeI* and *EcoRI* and ligation into the pET24 vector (Invitrogen). The resulting expression plasmid, pET24:*genK*, was used to transform *E. coli* Rosetta DE3 (Novagen) for expression of native, recombinant GenK. Transformants were grown in 3 L of LB medium at 18 °C with shaking at 200 rpm. GenK expression was induced with 1 mM IPTG

when the cells had reached an optical density of 0.6 at 600 nm. Cells were harvested by centrifugation 20 hr after induction (5000 × *g*, 15 min), resuspended in 50 mM Tris•HCl buffer (pH 8.0) containing 10% glycerol, and 1 mM PMSF, and lysed by sonication. Cell lysate was centrifuged at 25,000 × *g* for 30 min to pellet the insoluble material. Preliminary protein expression analysis using SDS-PAGE revealed that GenK, although abundant, was expressed exclusively as inclusion bodies. Therefore, the resulting supernatant was discarded and the pelleted inclusion bodies were washed three times in lysis buffer and dissolved in 20 mL of 50 mM Tris•HCl buffer (pH 8.0) containing 20% glycerol, 1 mM PMSF and 5 M urea. This material was centrifuged at 4,000 × *g* for 10 min to remove insoluble material.

2.3. *Refolding of GenK from Inclusion Bodies and Reconstitution with Iron and Sulfide*

The solublized GenK inclusion bodies, processed as described above, were added dropwise to 200 mL of refolding buffer composed of 50 mM Tris•HCl (pH 8.0), 1 mM $MgSO_4$, 5 mM reduced glutathione, 0.5 mM glutathione disulfide, 5 mM DTT, and 10% glycerol. This mixture was kept refrigerated for 24 hr with gentle stirring and was then concentrated to 7 mL in an Amicon YM-10 centrifugal concentrator. The protein was dialyzed overnight against 800 mL of 50 mM Tris•HCl (pH 8.0) containing 0.1 M NaCl, 1 mM DTT, and 20% glycerol. Afterward, the protein was centrifuged to remove precipitated material and the supernatant was reconstituted with iron and sulfide in an anaerobic glovebox according to an established procedure.[4] Iron content in the reconstituted GenK was assessed using the ferrozine assay[5] and the sulfide content was determined by the method devised by Helmut Bienert.[6] The iron and sulfide content reported in the article is an average of three measurements.

2.4. *Enzymatic Synthesis and Purification of S-adenosyl-L-methionine (SAM)*

The gene encoding *E. coli* SAM synthetase (*metK*) was cloned into pET28b(+) and the resulting construct was used to transform BL21(DE3) for expression of recombinant His_6-tagged protein. Expression of 6 L of *E. coli* containing pET28b(+):*metK* in LB media was induced with 0.1 mM IPTG when the culture had reached an optical density of 0.5 at 600 nm, grown overnight at 25 °C, harvested by centrifugation (5000 × *g*, 15 min), and resuspended in 100 mL of 50 mM Tris•HCl (pH 8.0), 1 M NaCl, 1 mM β-mercaptoethanol, 10 mM imidazole, and 10% glycerol (lysis buffer). Resuspended cells were lysed by sonication and the lysate was centrifuged at 27,200 × *g* for 20 min. Cleared lysate was mixed with 10 mL of Ni^{2+}-nitrilotriacetic acid (NTA) resin (Qiagen) pre-equilibrated with wash buffer (lysis buffer containing 20 mM imidazole) for 90 min. The Ni^{2+}-NTA was poured into a column casing and rinsed with 100 mL of the wash buffer. MetK was eluted by the addition of elution buffer (lysis buffer containing 0.25 M imidazole). The pooled MetK was dialyzed against 1 L of 50 mM Tris•HCl (pH 8.0), 0.15 M NaCl, 1 mM β-mercaptoethanol, and 10% glycerol. The final yield of MetK was 60 mL of 9.5 mg/mL. MetK was used to prepare SAM enzymatically from L-methionine and ATP and purified as described previously.[7] $^{13}CD_3$-*methyl*-SAM prepared from $^{13}CD_3$-*methyl*-L-methionine (purchased from SigmaAldrich) using the same procedure. The purity of the enzymatically prepared SAM was confirmed by high performance liquid chromatography (HPLC) using a Dionex CarboPac PA1 (4 × 250 mm) column with an isocratic 0.5 M ammonium acetate elution at 1 mL/min. Pure SAM was lyophilized, resuspended in 2 mL H_2O, divided into 50 μL aliquots, and stored at –80 °C until use.

2.5. *GenK Activity Assays*

The GenK activity assays were conducted at ambient temperature under anaerobic conditions in a Coy anaerobic chamber. The reaction mixture contained the following: 50 mM Tris•HCl (pH 8.0), 10 mM DTT, 1 mM MV, 4 mM NADPH, 4 mM enzymatically prepared SAM, 1 mM $GenX_2$ (**7**), 1 mM Cbl, and 5 μM GenK (unless otherwise noted). Assays were initiated by the addition of $GenX_2$. The reducing system for one of the reactions depicted in Figure S4 was 4 mM NADPH, 100 μM *E. coli* flavodoxin, 100 μM *E. coli* flavodoxin reductase (obtained as described[8]) rather than NADPH and MV.

Portions of the assays designated for HPLC detection of the adenosylated products, SAH (**5**) and 5′-dAdo (**4**), were quenched by adding a volume of 30% TCA equal to one tenth of the analyte volume.

Portions of the assays designated for detection of aminoglycosides (30 μL) were quenched with a mixture containing 112 μL of methanol, 6 μL of 0.5 M NaOH, and 1.8 μL of 8.4 M DFNB to give final concentrations of 20 mM NaOH, 75% methanol, and 0.1 M DNFB. These were heated at 80 °C for 5 min to promote derivatization of primary amines with DFNB so they could be detected by UV absorbance at 340 nm.[9]

Assays analyzed by mass spectroscopy were quenched by passage through YM-10 Microcon centrifugal filters to remove GenK.

2.6. *HPLC Detection of SAH (**5**) and 5′-dAdo (**4**)*

A 25 μL aliquot of the TCA-quenched GenK reaction described above was analyzed using HPLC equipped with a Varian Microsorb-MV 100-5 C18 (4.6 × 250 mm) column pre-equilibrated in H_2O containing 0.1% trifluoroacetic acid (solvent A). After sample loading, the column was washed with a linear gradient from 0–20% acetonitrile containing 0.1% trifluoroacetic acid (solvent B) over 30 min to resolve 5′-dAdo and SAH. The UV detector was set at 260 nm. A blend of authentic, commercially obtained 5′-dAdo and SAH was injected as a retention time standard for each. Quantitation of 5′-dAdo and SAH produced during assays was achieved by comparison of analyte peak areas with those of 5′-dAdo and SAH standards of known concentration.

2.7. *HPLC Detection of Aminoglycosides*

A 10 μL aliquot of DNFB-derivatized GenK assay mixtures prepared as described in section **2.5** was loaded onto a Varian Microsorb-MV 100-5 C18 (4.6 × 250 mm) column and eluted with a gradient from 60:40 → 50:50 water:acetonitrile over 20 min. The UV detector was set at 340 nm. A blend of authentic, commercially obtained $GenX_2$ (**7**) and G418 (**8**) was injected as a retention time standard for each. Quantitation of the conversion of $GenX_2$ to G418 was achieved by comparing the peak areas for each.

2.8. *Mass Spectroscopic Analysis of GenK Activity*

Assay mixtures were diluted in methanol and injected directly into an Agilent 6530 Accurate Mass QTOF-MS at 0.15 mL/min. The analyte was ionized by electrospray ionization and detected in positive ion mode.

S3. Supporting Figures

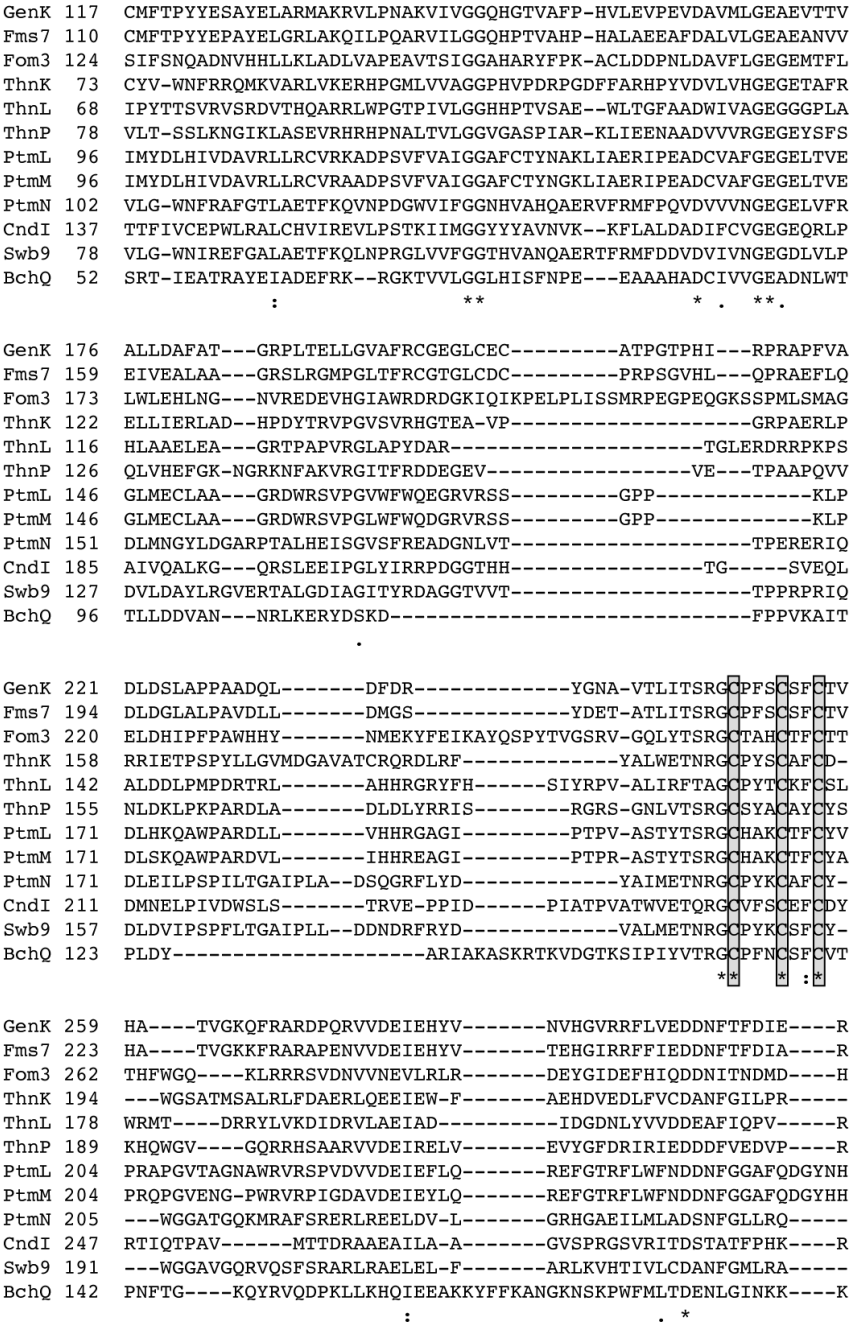

Figure S1. Partial amino acid sequence alignment of demonstrated and putative Cbl-dependent radical SAM sp^3-carbon methyltransferases. The absence of a conserved histidine residue within the Cbl-binding domain indicates that the corrin cobalt is probably coordinated to dimethylbenzimidazole. This is in contrast to methionine synthase, methyl malonyl-CoA mutase, glutamate mutase and others in which histidine in a conserved $DXHX_2G$ motif serves as the lower axial ligand for the corrinoid cobalt.[10] The sequence alignment also suggests that Cbl-dependent radical SAM sp^3 *C*-methyltransferases harbor a single [4Fe-4S] cluster per monomer as only the cysteine residues comprising the CX_3CX_2C motif (highlighted in grey) are universally conserved in this class of enzymes. GenK (gi|85814024) from *Micromonospora echinospora*, Fms7 (gi|1125024) from *Micromonospora olivasterospora*, Fom3 (gi|196166519) from *Streptomyces fradiae*, ThnK (gi|30577688), ThnL (gi|30577689), and ThnP (gi|30577693) from *Streptomyces cattleya,* PtmL (gi|212379254), PtmM (gi|212379255), and PtmN (gi|212379256) from *Streptomyces pactum*, CndI (gi|223940938) from *Chondromyces crocatus*, Swb9 (gi|283131230) from *Streptomyces* sp. SNA15896, and BchQ (gi|21674591) from *Chlorobium tepidum* TLS are involved in the biosynthesis of gentamicin, fortimicin, fosfomycin, thienamycin, pactamycin, chondrochlorens, quinomycin, 2-methylhopanoid, and bacteriochlorophyll biosynthesis, respectively.

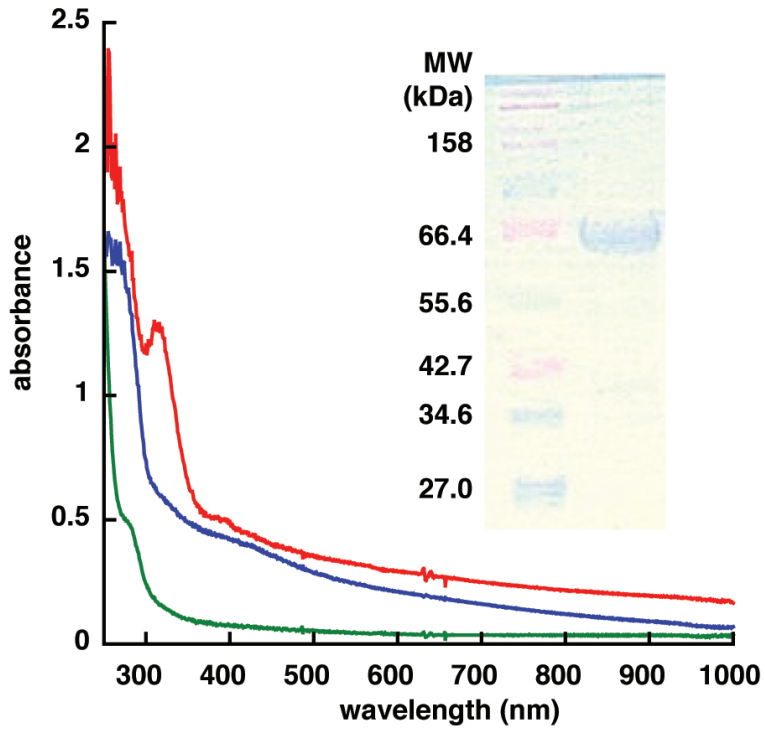

Figure S2. UV-visible absorbance spectra and SDS PAGE of GenK. The green, blue, and red spectra correspond to unreconstituted GenK (8 mM), reconstituted GenK (15 mM), and reconstituted (15 mM) GenK treated with 1 mM sodium dithionite. The bleaching of the absorbance shoulder at 420 nm is characteristic of a bound iron-sulfur cluster. The calculated molecular weight of GenK is 69.3 kDa.

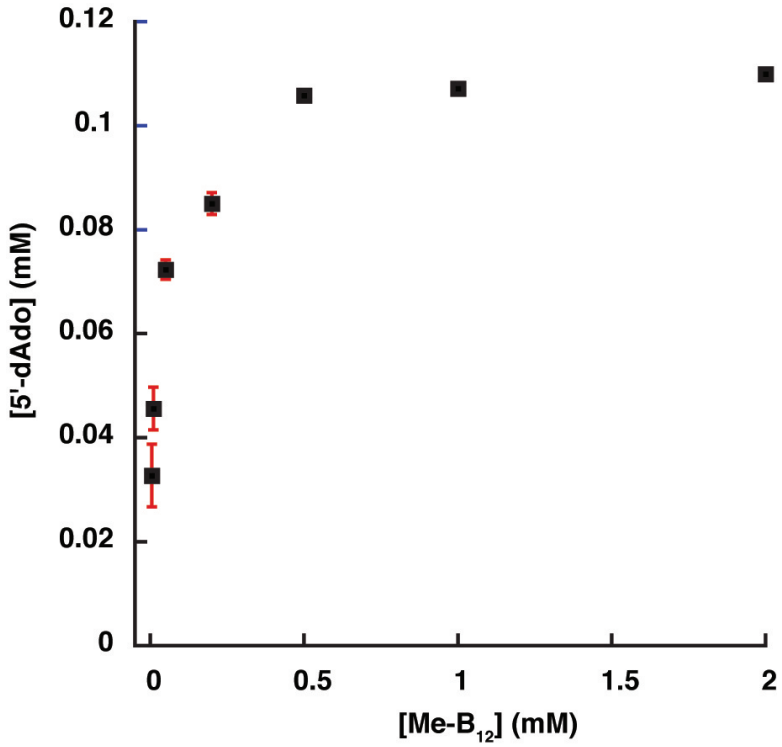

Figure S3. GenK activity as a function of [MeCbl]. Based on these results, 1 mM Cbl was used during the GenK activity assays described herein.

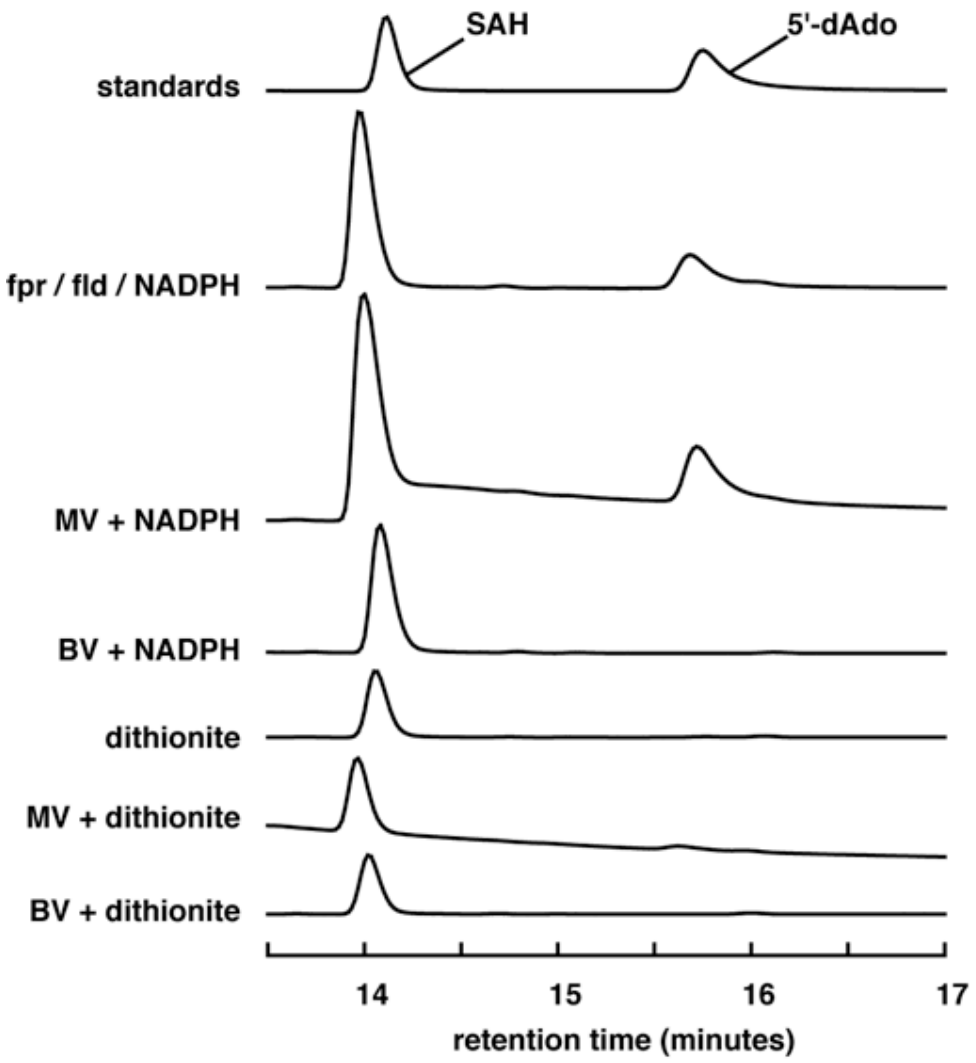

Figure S4. Effectiveness of various reducing systems for activation of GenK. HPLC traces with UV detection at 260 nm for observation of SAH and 5′-dAdo are depicted. Among the experimental conditions, only MV and NADPH or the flavodoxin/flavodoxin reductase/NADPH (fpr/fld/NADPH) system were capable of activating GenK. Although SAH is present in all traces, separate experiments demonstrated that it is formed non-enzymatically due to the methylation of reduced cobalamin under the reaction conditions employed.

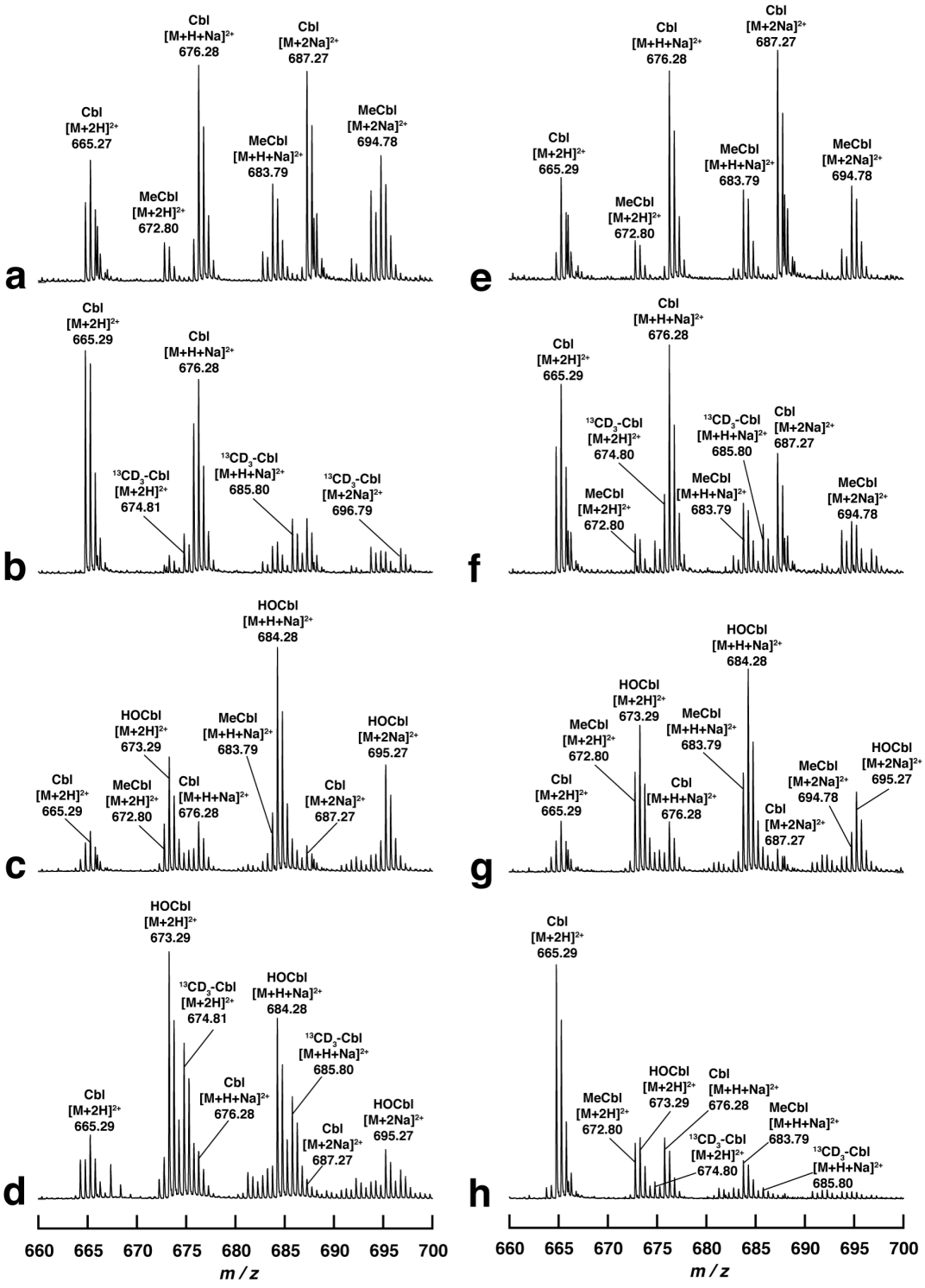

Figure S5. Mass spectra of cobalamin displaying incorporation of methyl from SAM and/or $^{13}CD_3$ from $^{13}CD_3$-*methyl*-SAM into hydroxocobalamin and methylcobalamin. The assays are the same as those shown in Fig. 3 but the range of *m/z* values corresponds to the region in which Cbl^{2+} is located. The $^{13}CD_3$-Cbl signal is more visible when spectrum h was recorded under the $[M+H]^+$ mode (data not shown).

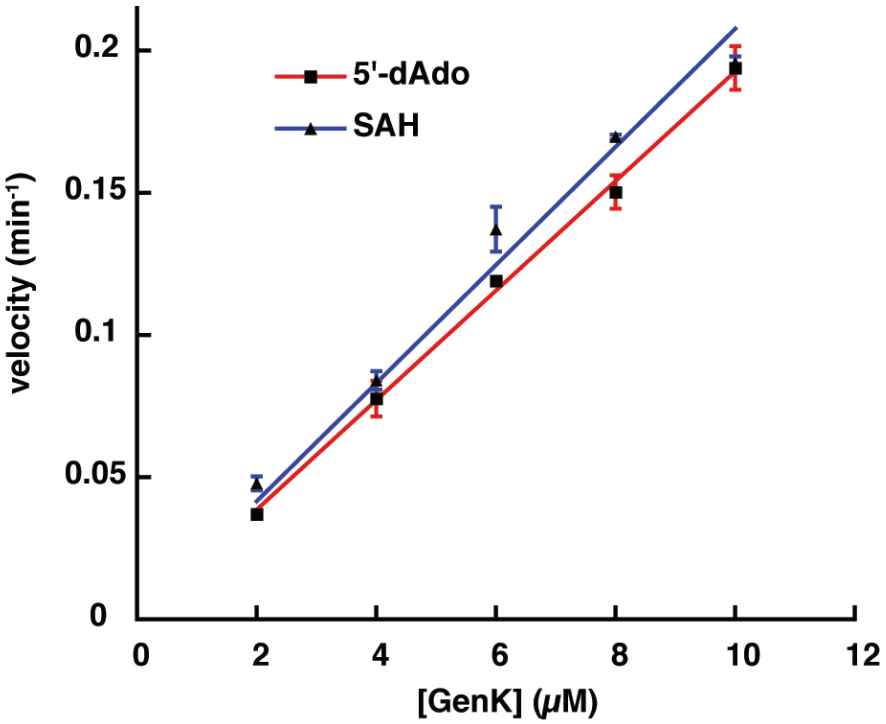

Figure S6. Reaction velocity as a function of [GenK]. Reactions were quenched at 8 hr (previous experiments showed that, under the conditions employed, the rate of catalysis remains constant over this timescale, see Fig 4). Reactions were conducted in duplicate. Control reactions without GenK were used to assess the quantity of non-enzymatically produced SAH and this amount was subtracted out of the enzyme assay results to give the data shown here. The rate of both 5′-dAdo and SAH production during this experiment is about ~0.02 min^{-1}.

S.4. References

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